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NKCC1 downregulation induces hyperpolarizing shift of GABA responsiveness at near term fetal stages in rat cultured dorsal root ganglion neurons

Joelle N Chabwine^{1,2*}, Karel Talavera³, Ludo Van Den Bosch^{4,5} and Geert Callewaert¹**Abstract**

Background: GABA_A receptor-mediated neurotransmission is greatly influenced by cation-chloride cotransporter activity during developmental stages. In embryonic neurons Na–K–2Cl (NKCC1) cotransporters mediate active chloride uptake, thus increasing the intracellular chloride concentration associated with GABA-induced depolarization. At fetal stages near term, oxytocin-induced NKCC1 downregulation has been implicated in the developmental shift from depolarizing to hyperpolarizing GABA action. Mature dorsal root ganglion neurons (DRGN), however, express high NKCC1 levels and maintain high intracellular chloride levels with consequent GABA-induced depolarization.

Results: Gramicidin-perforated patch-clamp recordings were used to assess the developmental change in chloride homeostasis in rat cultured small DRGN at the embryonic day 16 (E16) and 19 (E19). The results were compared to data previously obtained in fetal DRGN at E14 and in mature cells. A significant NKCC1 downregulation, leading to reduction in excitatory GABAergic transmission, was observed at E16 and E19.

Conclusion: These results indicate that NKCC1 activity transiently decreases in DRGN at fetal stages near term. This developmental shift in GABAergic transmission may contribute to fetal analgesia and neuroprotection at birth.

Keywords: Dorsal root ganglion neurons, GABA, Intracellular chloride, NKCC1, Bumetanide, Oxytocin, Fetal analgesia

Background

Cation-chloride cotransporters largely determine the action of GABA during neurogenesis [1–5]. In immature neurons, Na–K–2Cl (NKCC1) mediates active Cl[−] uptake, promoting depolarizing GABA action, whereas in adult neurons the Cl[−]-extruding KCC2 is generally considered to be involved in generating the hyperpolarizing effect of GABA. Mature dorsal root ganglion neurons (DRGN), however, express high NKCC1 levels and maintain high intracellular Cl[−] concentration ([Cl[−]]_i) with resulting GABA-induced depolarization [2, 6, 7].

This property is crucial for presynaptic inhibition of spinal sensory feedback [2, 7–11].

A NKCC1 downregulation has been observed around birth in different neuronal types of central nervous system (CNS) [2, 12, 13] as a result of circulating maternal oxytocin [14–16]. Previously, we showed that DRGN at embryonic day 14 (E14) displayed higher NKCC1 activity and higher intracellular [Cl[−]]_i levels than age-matched motor neurons (MN) [17]. Here, we investigated whether DRGN also display NKCC1 downregulation at fetal stages near term, before increasing again to high expression levels in adult stage [18, 19].

Our data show a marked decrease in DRGN [Cl[−]]_i at E16 and E19 compared to E14. Decreased activity of NKCC1 at E16 and E19 fully accounts for this reduction in [Cl[−]]_i. A possible role for this transient shift in GABAergic responses in DRGN is discussed.

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Results and discussion

GABA-induced currents were measured in cultured E16 and E19 DRGN. The results were quantitatively compared to data previously obtained in DRGN at E14 [17].

Total membrane capacitance remained stable between E14 [17] and E16 at 24 ± 2 pF ($n = 11$, $p = 0.43$), and slightly increased to 30.7 ± 2 pF at E19 ($n = 12$, $p = 0.006$), indicating that most of the cells examined were small DRGN (capacitance $30 \leq$ pF) [11]. Based on E_{GABA} , $[Cl^-]_i$ markedly decreased from 44 mM at E14 to 30 and 29 mM at E16 and E19, respectively (Figure 1). In the presence of the selective NKCC1 blocker bumetanide (10 μ M), $[Cl^-]_i$ was significantly reduced to ~20 mM at all stages, indicating that NKCC1-dependent Cl^- influx significantly dropped between E14 (57%) and E16 (33%), with no further decrease up to E19 (38%).

Changes in NKCC1 regulation were further explored by studying $[Cl^-]_i$ recovery after Cl^- load and Cl^- depletion in DRGN at E19. Applying 1.5 mM GABA for 5 s at a membrane potential of +70 mV consistently shifted the reversal potential in the positive direction, indicating an increase in $[Cl^-]_i$ from 33 to 43 mM ($n = 2$). Similar results were previously obtained in E14 DRGN [17] ($p = 0.38$ between respective increases in $[Cl^-]_i$). Following Cl^- loading, recovery to basal $[Cl^-]_i$ levels followed a single exponential function with a time constant of 2.97 ± 0.1 min ($n = 5$, Figure 2b), which is not different from that previously recorded in E14 DRGN [17] (Figure 2a, $p = 0.44$). Applying 1.5 mM GABA for 5 s at a membrane potential of -100 mV reduced $[Cl^-]_i$ by only 3 mM [from 31 to 28 mM ($n = 3$)] whereas in

E14 DRGN, $[Cl^-]_i$ dropped by 8 mM. This difference in $[Cl^-]_i$ reduction between E14 and E19 DRGN during the depletion protocol was significant ($p = 0.009$). Following Cl^- depletion, recovery to the resting $[Cl^-]_i$ level was also significantly slower in E19 DRGN (time constant of 1.64 ± 0.1 min, $n = 5$) (Figure 2d) than in E14 DRGN (time constant of 0.9 ± 0.1 min) ($p = 0.002$, Figure 2c). Together, these data strongly suggest that NKCC1-related Cl^- fluxes markedly decreased in small DRGN after day E14.

It was previously shown that mature and embryonic DRGN sustain high $[Cl^-]_i$ owing to high NKCC1 expression [2, 11, 18]. Here, we show that embryonic DRGN undergo a significant downregulation of NKCC1 between E14 and E16 (maintained through E19), which significantly affects Cl^- homeostasis. The resulting overall decrease in $[Cl^-]_i$ limits the depolarizing action of GABA. Notably, $[Cl^-]_i$ at E14 is comparable to the level reported in the postnatal period (P0–P21) [19, 20] and possibly in adult DRGN [18] implying that the observed drop in $[Cl^-]_i$ between day E16 and E19 is only a transient phenomenon. The underlying mechanism most probably involves decreased NKCC1 activity rather than lower expression level [14, 20–22].

Conclusion

These data accord with previous observations in other CNS neuronal types showing that NKCC1 activity is decreased as a result of increasing levels of circulating maternal oxytocin at near term. However, in contrast to other neuronal types, the activity-dependent downregulation of NKCC1 in DRGN is not permanent but only transient. Since we predominantly studied small DRGN (capacitances ≤ 30 pF) [11] in which GABA-induced depolarization is essential for sensory perception [2, 7–9, 11], we suggest that the transient GABAergic hyperpolarizing shift observed between E16 and E19 reflects oxytocin-induced fetal adaptation to delivery [14, 16], contributes to fetal analgesia and protects the fetus against neuronal insult.

Methods

All experimental procedures were approved by the local Ethical Committee and were therefore performed in accordance with international ethical regulations. DRGN were derived from Wistar rat embryos at E16 and E19, and cultured as previously described [17]. Tissue samples were trypsinized and triturated and neurons were purified by centrifugation using a bovine serum albumin cushion and then plated on poly-L-ornithine and laminin-coated glass coverslips. Neurons were used between 3 and 6 days after plating. GABA-induced currents (I_{GABA}) were recorded under voltage-clamp conditions using

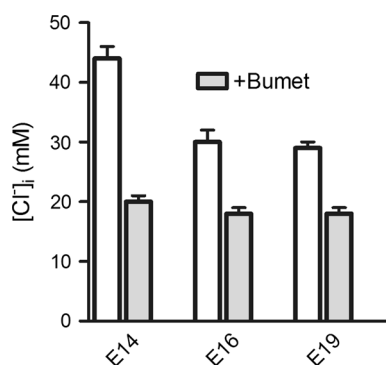
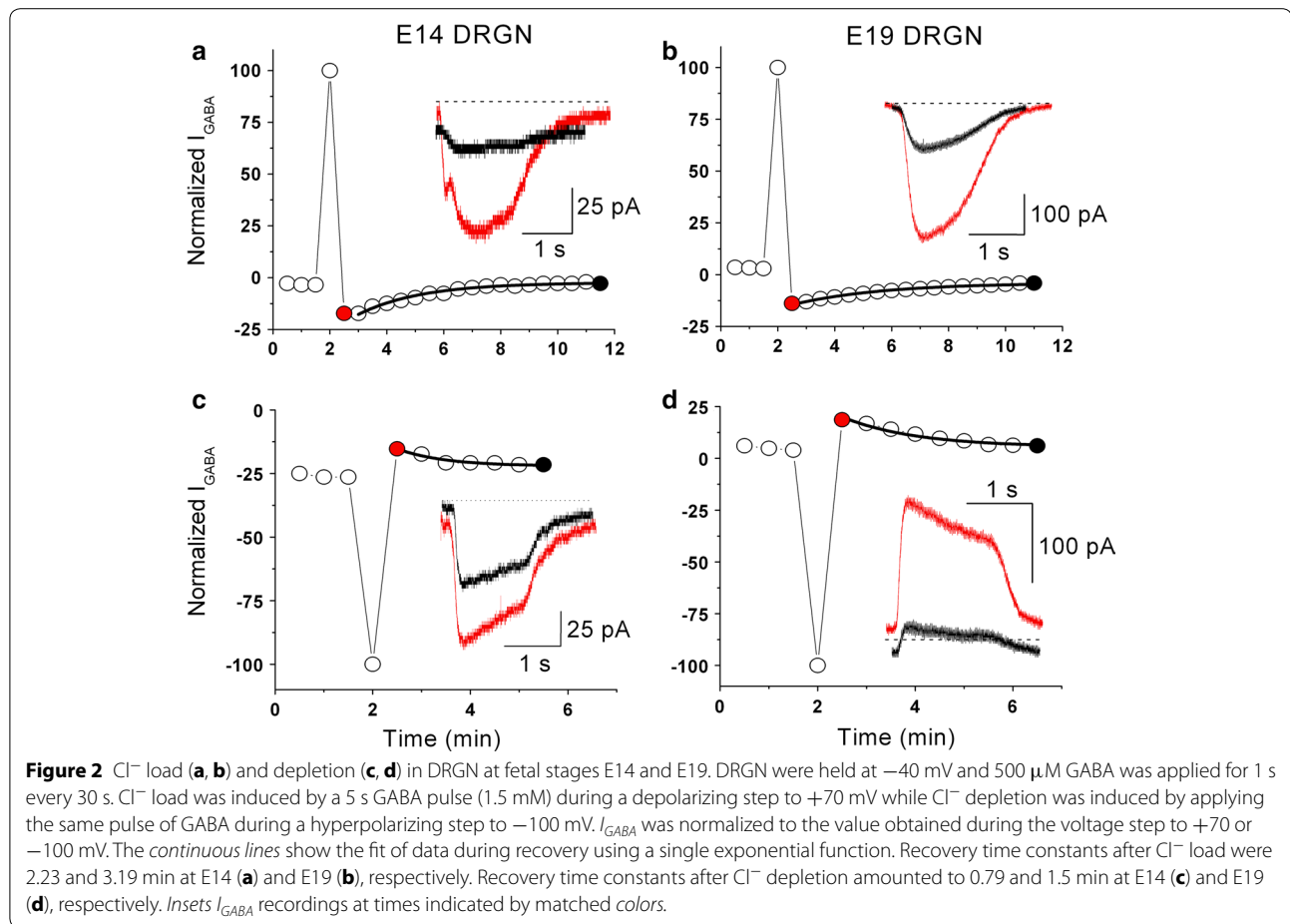


Figure 1 Intracellular $[Cl^-]_i$ and bumetanide-induced Cl^- reduction in DRGN. The resting $[Cl^-]_i$ (open bars) dropped significantly between E14 (44 ± 2 mM, $n = 71$) and E16 (30 ± 2 mM, $n = 11$, $p = 0.00003$), with no further decrease at E19 (29 ± 1 mM, $n = 13$, $p = 0.81$). In the presence of 10 μ M bumetanide (+Bumet, grey bars), a specific blocker of NKCC1, $[Cl^-]_i$ was decreased to 20 ± 1 mM ($n = 6$) at E14 and to 18 ± 1 mM ($n = 6$) at E16 and E19. The bumetanide-insensitive component remained stable at all stages ($p = 0.73$ for E14 versus E16 and $p = 0.46$ for E14 versus E19).



gramicidin-perforated patches [17] ($75 \mu\text{g/ml}$, Fluka). The pipette solution contained (in mM): CsCl 125, MgCl_2 1.2, HEPES 10, Na_2ATP 2 and EGTA 1 and the pH was adjusted to 7.3 with CsOH. Patch pipettes ($3\text{--}5$ M Ω resistance) were briefly dipped in gramicidin-free pipette solution and back-filled with internal solution. After seal formation and stable access resistance ≤ 30 M Ω , GABA was locally applied using a fast perfusion system. The external solution contained (in mM): NaCl 150, KCl 6, MgCl_2 1, CaCl_2 3, HEPES 10 and glucose 10, pH adjusted to 7.3 with NaOH. 500 nM TTX (tetrodotoxin, Alomone labs) and $100 \mu\text{M}$ Cd^{2+} were added to block voltage-gated Na^+ and Ca^{2+} channels, respectively. Total external $[\text{Cl}^-]$ amounted to 164 mM. Bumetanide ($10 \mu\text{M}$) was used to specifically block NKCC1 cotransporters [4, 6].

The reversal potential for I_{GABA} , (E_{GABA}) determined with brief voltage ramps during GABA application, was used to estimate intracellular Cl^- levels [17]. Intracellular $[\text{Cl}^-]$ depletion or loading was achieved by applying a 5 s pulse of GABA (1.5 mM) during a voltage step from the holding potential (-40 mV) to -100 mV or to $+70$ mV,

respectively [12]. After depletion or loading, $[\text{Cl}^-]$ recovery was monitored by applying successive 1 s-GABA pulses of $500 \mu\text{M}$ every 30 s. [17]. All data are shown as mean \pm SEM. Statistically significant differences were evaluated with unpaired Student t test ($p < 0.05$).

Abbreviations

$[\text{Cl}^-]_i$: intracellular chloride concentration; CNS: central nervous system; DRGN: dorsal root ganglion neuron; E_{GABA} : reversal potential for I_{GABA} ; EGTA: ethylene glycol tetraacetic acid; E14: embryonic day 14; E16: embryonic day 16; E19: embryonic day 19; GABA: gamma amino butyric acid; HEPES: 4-(2-Hydroxy-ethyl)piperazine-1-ethanesulfonic acid; I_{GABA} : GABA-induced current; KCC2: potassium-chloride (K-Cl) cotransporter 2; MN: motor neuron; NKCC1: sodium-potassium-chloride (Na-K-2Cl) cotransporter 1; P0: post-natal day 0; P21: post-natal day 21; TTX: tetrodotoxin.

Authors' contributions

JNC designed the study, carried all experiments, analyzed data, drafted and revised the manuscript. KT significantly contributed to the conception and the design of the study, to data analysis and to the revision of the manuscript. LVDB contributed to data acquisition, data analysis and manuscript revision. GC supervised the whole study, significantly contributed to its conception and experimental design, to data acquisition and analysis, and to manuscript revision. All authors read and approved the final manuscript.

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Compliance with ethical guidelines**Competing interests**

The authors declare that they have no competing interests.

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